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Research Article

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ABSTRACT Intravenous cationic bovine serum albumin (BSA, pI > 9.5) induces membranous nephropathy in immunized rabbits. In this study, unimmunized rabbits received intravenous injections of cationic ($n = 3$) or native ($n = 3$) BSA, followed by ex vivo isolated left renal perfusions with sheep anti-BSA antibody. Capillary wall deposits of IgG and C3 were seen exclusively in the group receiving cationic BSA, confirming an in situ pathogenesis for cationic, BSA-induced membranous nephropathy, and demonstrating the importance of a cationic antigen for its production. We then explored whether membranous nephropathy in this model is prevented by the concomitant injection of protamine sulfate, a filterable, relatively non-immunogenic polycation. An in vitro study demonstrated that protamine sulfate incubated with glomerular basement membrane (GBM) decreased the subsequent binding of radiolabeled cationic BSA ($P < 0.05$). In vivo, protamine sulfate was shown to bind to anionic sites in the glomerular capillary wall after intravenous injection.

Groups of rabbits received 3 wk of daily intravenous injections of cationic BSA alone ($n = 15$) or cationic BSA and protamine ($n = 18$). After 2 wk of injection of cationic BSA alone, typical membranous nephropathy developed. Granular deposits of IgG and C3 were present along the GBM associated with subepithelial dense deposits, foot process effacement, and marked albuminuria. Protamine significantly reduced or prevented the formation of deposits ($P < 0.001$) and in 6 of 18 protamine-treated animals, existing deposits decreased or disappeared between 2 and 3 wk of injection. Albuminuria was significantly reduced in protamine-treated animals with a mean of 124±55 mg/24 h compared to 632±150 mg/24 h in the control group receiving cationic BSA alone. No significant differences between the groups were noted in serum levels of IgG, C3, anti-BSA antibody, or circulating immune complex size. Studies in additional animals ($n = 5$) given radiolabeled cationic BSA showed that protamine did not alter the clearance of cationic BSA from serum.

Control experiments showed that protamine's beneficial effects were not related to its weak anticoagulant property or to its theoretical ability to deplete tissue histamine. The administration of heparin ($n = 6$) or diphenhydramine ($n = 6$) had no effect on the development of the epimembranous lesion compared to the group receiving cationic BSA alone. In addition, homogenized whole kidney histamine content was not significantly different in the group receiving cationic BSA alone compared to the group receiving cationic BSA and protamine.

This work shows that a cationic BSA-induced glomerular lesion can be produced by a renal perfusion technique involving in situ complex formation and that this process requires a cationic antigen for its de-

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velopment. We believe that the demonstrated beneficial effects of protamine are due to its ability to bind to glomerular anionic sites, and that this electrostatic interaction results in inhibition for the further binding of the cationic antigen, thereby limiting the severity of glomerulonephritis in this model.

INTRODUCTION

Membranous nephropathy is the most common cause of the idiopathic nephrotic syndrome in adults. In recent years, evidence has been accumulating that supports the hypothesis that the subepithelial immune deposits seen in membranous nephropathy are formed in situ, that is, an exogenous antigen or an antigen intrinsic to the glomerular basement membrane (GBM)[1] combines locally with an antibody to form an immune complex (1). Early support for in situ complex formation was provided by Van Damme et al. (2) who showed that renal lesions could be produced in the passive Heymann nephritis model in the absence of circulating immune complexes by the in vivo perfusion of rat kidneys with rabbit anti-rat FxlA. Further evidence that the subepithelial deposits seen in passive and active Heymann nephritis could be reproduced by the in vivo perfusion method and were therefore not dependent on the presence of circulating immune complexes was submitted by Couser et al. (3). Together, these studies introduced the idea that intrinsic renal antigens may play a role in the development of in situ immune complex formation in the kidney. Later work from other laboratories showed that in situ complex formation in the rat kidney could also result from GBM binding of exogenous antigens. The work of Golbus and Wilson using concanavalin A (4), Fleuren et al. using bovine serum albumin (BSA) (5), and Batsford et al. (6) using cationized ferritin (6), all demonstrated that the administration of exogenous antigen followed by antibody could result in in situ immune complex formation in the kidney.

Native BSA, with a pI 4.9, has been used extensively in the production of acute and chronic serum sickness in the rabbit (7, 8). Recent work from our laboratory involved the development of a highly predictable and reproducible model of membranous nephropathy in the rabbit utilizing charge-modified cationic BSA (pI > 9.5) as the antigen (9). In this model, animals preimmunized to cationic BSA developed capillary wall immune deposits consisting of cationic BSA, IgG, and C3 after 2 wk of intravenous injections of cationic BSA, progressing in severity over a 6-wk injection period. The immunofluorescence findings were accompanied by the presence of subepithelial dense deposits by electron microscopy and by "spike" formation along the capillary wall by light microscopy after staining with silver methenamine. Albuminuria developed in these animals in association with the observed morphologic abnormalities. These pathologic findings parallel the lesions seen in idiopathic membranous nephropathy in man.

It is probable that the subepithelial deposits observed in the cationic BSA model are initiated by a primary electrostatic attraction between cationic BSA and the fixed anionic sites of the glomerular capillary wall described by Caulfield and Farquhar (10) and Kanwar and Farquhar (11). In the current study, we used an ex vivo perfusion technique to demonstrate the in situ pathogenic mechanism of cationic BSA-induced, membranous nephropathy. In addition, we explored the hypothesis that a small, highly cationic, relatively nonimmunogenic molecule, freely filterable at the glomerulus, if given concomitantly with the disease-producing cationic BSA, would compete for binding at these anionic sites and effectively prevent or delay the development of membranous nephropathy. Protamine sulfate is a substance whose properties of weak immunogenicity, highly cationic charge (pI > 10), low molecular weight (7,000), and commercial availability made it ideal to test this hypothesis.

METHODS

Perfusion experiments. The purpose of these perfusion studies was to establish the pathogenic mechanism involved in the development of the immune deposits induced by the injection of cationic BSA. Two groups of three New Zealand white rabbits (unimmunized) weighing 2–3 kg were studied. Group A (n = 3) received a single injection of cationic BSA 25 mg i.v. and group B (n = 3) received native BSA 25 mg i.v. 15 min after the injection of the respective BSA, animals were anesthetized with Xylazine 30 mg i.m. (Cutter Laboratories, Shawnee, KS) and Ketamine HCl 70 mg i.m. (Parke-Davis Co., Detroit, MI). A midline abdominal incision was made and the left kidney exposed. A polyethylene catheter was placed in the left renal artery, which was ligated distal to the origin of the left spermatic and adrenal arteries. The left renal vein was punctured and 37°C normal saline (3.7 ml) was perfused for 1 min, at which time the left kidney was bloodless. Sheep anti-BSA antibody was then perfused over 1 min through the left kidney and removed from the abdomen by absorption with cotton swabs. After antibody perfusion, the kidney was perfused with 37°C normal saline to remove any unbound antibody from the kidney. Biopsies were taken from the left kidney and from the right unperfused kidney as a control. Total ischemia time up to this point was <10 min. After these biopsies, the left renal artery and vein were repaired by compression with a hemostatic gelatin sponge and circulation reestablished. Repeat renal

[1] *Abbreviations used in this paper*: BSA, bovine serum albumin; FxlA, fraction 1A derived from renal tubular epithelium; GBM, glomerular basement membrane; NRS, heat-inactivated 10% normal rabbit serum; PBS, phosphate-buffered saline.

TABLE I
Perfusion Protocol

Group	n	Intravenous antigen injection	Antibody perfused*
A	3	Cationic BSA, 25 mg	Sheep anti-BSA
B	3	Anionic BSA, 25 mg	Sheep anti-BSA

* Before and immediately after the 1-min antibody perfusion, the kidney was perfused with normal saline, initially to wash out blood, and finally to remove unbound antibody.

biopsies were done 10–20 min after reestablishment of the circulation (Table I).

Preparation of BSA antigen and anti-BSA antibody. Native crystalline BSA (Miles Laboratories, Elkhart, IN) was used unmodified as native BSA (pI 4.9) or was cationized to a pI > 9.5, lyophilized and stored at −20°C as described in detail previously (9). The isoelectric point of the resulting cationic BSA were confirmed by isoelectric focusing in thin-layer agarose gels with ampholines, pH 3.5–9.5, using an LKB flatbed electrofocusing unit (LKB Instruments, Inc., Rockville, MD). The pH gradient was measured directly from the gels using an LKB surface glass electrode and a Beckman pH meter (Beckman Instruments, Inc., Palo Alto, CA). Focusing was repeated every 2 wk for 6 wk to demonstrate the stability of the charge modification. Lyophilized cationic BSA was dissolved in phosphate-buffered saline (PBS) each day just before injection and placed in a Beckman centrifuge model TJ-6 at 1,000 *g* at 4°C for 30 min to remove insoluble aggregates.

Sheep anti-BSA antibody was obtained after separate sequential subcutaneous immunizations of a single sheep with both cationic and native BSA 10 mg dissolved in 0.5 ml PBS and mixed with 0.5 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). Injections were repeated every 2 wk. Just before each boosting immunization, the animal was bled from the jugular vein and serum collected and stored at −20°C. Before use, 75 ml of antiserum was thawed and mixed with an equal volume of saturated ammonium sulfate for 1 h. The mixture was centrifuged for 30 min at 1,000 *g*, and the supernatant discarded. The precipitate was dissolved in 75 ml normal saline, once again mixed with saturated ammonium sulfate for 1 h, and centrifuged. The final precipitate was dissolved in normal saline, dialyzed for 48 h against borate buffer, pH 8.4, and centrifuged at 1,000 *g*, 4°C for 30 min just prior to use to remove aggregates. The antibody perfusate was shown by an Antigen Binding Capacity-33 test (19) to bind 1.1 mg BSA/ml.

In vitro inhibition study. An in vitro study consisting of sequential incubations of GBM with protamine followed by ^{125}I-cationic BSA was done to show that protamine is an inhibitor for the subsequent binding of cationic BSA. Glomeruli from bovine kidneys were isolated over sieves by the method of Spiro (12). Isolated glomeruli were sonicated, washed, and centrifuged to collect GBM by the method of Mohos and Skoza (13). Preliminary experiments showed that optimal cationic BSA binding by the GBM occurred with 5 μg cationic BSA and 200 μg GBM. In the primary experiment 200±7-μg aliquots of GBM were weighed on a Cahn 21 electrobalance (Cahn Instruments, Inc., Cerritos, CA) and placed in conical plastic tubes with 1 ml of protamine sulfate ($2.5–2.5 \times 10^{-3}$ mg/ml) or 1 ml of heat-inactivated 10% normal rabbit serum in PBS (to be referred to as NRS) or PBS alone. Six tubes at each dilution were done to increase accuracy. Duplicate tubes with identical amounts of protamine and NRS or PBS followed by ^{125}I-cationic BSA were used to control for the nonspecific binding of ^{125}I-cationic BSA to the walls of the tube. After addition of protamine and NRS or PBS, tubes were placed on a shaking platform at 4°C overnight. The next morning, centrifugation at 1,000 *g* for 30 min with removal of the supernatant was followed by two PBS washes, centrifugation, and discarding of the supernatant. 5 μg of ^{125}I-cationic BSA was then placed in each tube and a second overnight incubation at 4°C on a shaking platform was done. An identical centrifugation and washing procedure was performed. Tubes were counted in a Beckman automatic gamma counter.

In vivo inhibition studies. 45 male New Zealand White rabbits (2–3 kg) were housed separately and fed pellet food and tap water. Rabbits were immunized with cationic BSA 1 mg and an adjuvant, S. typhosa 0901 lipopolysaccharide W (Difco Laboratories, Detroit, MI) 1 μg i.v., and assigned to one of four experimental groups (I–IV) as described in Table II. Daily injections of cationic BSA 25 mg i.v. were begun 1 wk after immunization in all rabbits in groups I–IV and continued for 3 wk. All animals in groups II–IV were injected concomitantly with controls from group I. There were no historical controls.

Group I animals (n = 15) were given cationic BSA each morning. Group II animals (n = 18) were given protamine sulfate (kindly supplied by Lilly Research Laboratories, Indianapolis, IN), 50 mg i.v., followed immediately by saline and cationic BSA each morning. A second injection of protamine sulfate, 50 mg i.v. was given each day 7 h later.

Protamine sulfate is a weak anticoagulant (14). In addition, it is known that the chronic administration of a polycation may induce depletion of tissue histamine (15). For these reasons, two additional groups were studied to assess the effect of anticoagulation (group III) and the role of histamine (group IV) in the diminution of epimembranous deposits seen with protamine injections. Group III animals (n = 6) were given cationic BSA along with heparin 1,700 U/kg i.v. (Elkins-Sinn, Inc., Cherry Hill, NJ) twice daily substituted for protamine. This heparin dose was chosen to provide adequate anticoagulation with partial thromboplastin times ranging from two to four times control values (16). Group IV animals (n = 6) were given cationic BSA along with diphenhydramine 15 mg/kg per day i.m. (Elkins-Sinn, Inc.) in two doses substituted for protamine. Renal biopsies in groups I–IV were done after 2 and 3 wk of injection. The afternoon protamine (group II) or heparin (group III) injections were omitted on the day prior to renal biopsy in order to minimize the risk of hemorrhage during surgery.

Two nonimmunized rabbits were assigned to an additional control group (V) to assess the effects of long term protamine injections on renal morphology and function. Group V rabbits received protamine sulfate, 50 mg i.v., twice daily for 6 wk. Additional animals (n = 5) were used to document protamine's ability to bind to anionic sites in the rabbit glomerulus after an intravenous injection. After anesthesia, the left kidney was isolated through a left flank incision. Rabbits then received normal saline 5 ml i.v. (n = 2) or protamine sulfate 50 mg i.v. (n = 3) infused over 5 min. 15 min after the beginning of the intravenous infusion, renal biopsies were taken and processed for electron microscopy. In order to demonstrate that serum levels of cationic BSA in protamine- and saline-pretreated animals were similar and that differences in morphology between groups I and II were not related to differences in serum cationic BSA levels, ^{125}I-cationic BSA, 0.25 mg i.v. was given to each of these five rabbits.

TABLE II
*Daily Injection Schedule**

Group	9 AM i.v. injection	4 PM i.v. injection
I (n = 15)	Cationic BSA, 25 mg	None
II (n = 18)	Protamine sulfate, 50 mg Cationic BSA, 25 mg	Protamine sulfate, 50 mg
III (n = 6)	Heparin, 3,500 U Cationic BSA, 25 mg	Heparin, 3,500 U
IV (n = 6)	Diphenhydramine, 15 mg i.m. Cationic BSA, 25 mg	Diphenhydramine, 15 mg i.m.
V (n = 2)	Protamine sulfate, 50 mg	Protamine sulfate, 50 mg

* 1 wk postimmunization, daily injections began and were continued for 3 wk. To prevent mixing of drugs in the intravenous tubing, a 3–5-ml normal saline flush separated infusions of each substance.

Blood was drawn through the central ear artery 0.25, 0.75, 2.5, and 24 h after the radiolabeled injection and serum was collected and counted in duplicate to measure cationic BSA levels.

Before open renal biopsy, animals were anesthetized with Xylazine, 30 mg i.m.; and Ketamine HCl, 70 mg i.m. 1% Lidocaine HCl (Cutter Laboratories, Shawnee, KA) was used as necessary. Nephrectomies were done in most animals at the end of 3 wk of injection, at which time kidneys were frozen and stored for further study. Surgical technique was clean, but not sterile.

Microscopic techniques. Tissue for immunofluorescence microscopy was snap frozen in isopentane and stored at −70°C. Frozen tissue was embedded in OCT compound (Lab-Tek Products, Naperville, IL) and cut into 4-μm sections, processed in a standard manner and stained with fluorescein-isothiocyanate conjugated antisera (Meloy Laboratories, Springfield, VA) to native BSA, rabbit IgG, rabbit C3, and rabbit albumin as described (9). The antiserum to native BSA reacted equally well with native and cationic BSA as determined by Ouchterlony analysis and by immunofluorescence examination of renal tissue containing BSA immune complex deposits. Slides were viewed on a Zeiss immunofluorescence microscope (Carl Zeiss, Inc., New York) equipped with epi-illumination and glomerular deposits were graded in a semiquantitative manner from 0 (negative) to 4 (maximal intensity) by an observer unaware of the tissue's group of origin.

Tissue for electron microscopy was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer or in a 2% phosphotungstic acid, 2% glutaraldehyde, 2% formaldehyde, 0.1 M cacodylate buffer mixture at a pH of 7.2 (17), post-fixed in 1% osmium tetroxide and processed as described (9).

Laboratory measurements. 24-h urine collections were done before immunization and at the end of 2 and 3 wk of injections. Quantitation of 24-h urinary rabbit albumin was done by the radial immunodiffusion technique (18) in agarose using goat anti-rabbit albumin (N. L. Cappel Laboratories, Cochranville, PA) and rabbit albumin standards (Miles Laboratories, Kankakee, IL).

All rabbits were bled via the central artery of the ear prior to immunization and 24 h after the previous dose of cationic BSA at the end of 2 and 3 wk of injections. Serum creatinine was measured using the Beckman creatinine autoanalyzer (Beckman Instruments, Inc.). Serum IgG and C3 were measured by radial immunodiffusion using goat anti-rabbit IgG and C3 in agarose; dilutions of purified rabbit IgG were used for standards (Cappel Laboratories). C3 results were expressed as the percentage of the value observed in each rabbit before immunization. Antigen Binding Capacity-33 measurements were done in a standard manner (19) using 2.5 μg ^{125}I-native BSA as the antigen at serum dilutions of 1:10, 1:50, and 1:250.

The molecular size of circulating immune complexes was determined as previously described (9) with minor modifications. Duplicate 50-μl serum aliquots were applied to linear 10–30% sucrose gradients. Molecular weight markers included radiolabeled IgM (Cappel Laboratories), aldolase (Pharmacia Fine Chemicals, Upssala, Sweden), glutamate dehydrogenase, and thyroglobulin (Sigma Chemical Co., St. Louis, MO). The samples were placed in an SW 50.1 rotor and spun at 100,000 g for 17 h at 4°C. Six drop fractions were collected and counted in a Beckman automatic gamma counter. Aliquots from ^{125}I-cationic BSA peaks were tested for the presence of IgG by double immunodiffusion. All radiolabeled proteins employed were iodinated by the chloramine T method resulting in specific activities as previously described (9).

Kidney histamine content. Early studies on the pathogenesis of serum sickness glomerulonephritis suggested that histamine played a role in immune complex deposition since it increased vascular permeability and is released during acute inflammatory processes (20). It has also been suggested that the chronic administration of polycations may induce depletion of tissue histamine (15). To show that the chronic administration of protamine sulfate along with cationic BSA did not deplete histamine and in this way affect the development of subepithelial deposits, we measured free histamine levels in homogenized whole kidney from three animals of group I and three of group II after 3 wk of injections. The fluorometric method used was previously described in detail by Shore et al. (21). 1 g of frozen whole kidney was homogenized in 5% trichloracetic acid and centrifuged; 1 ml of clear supernatant was assayed in duplicate for free histamine. Histamine standards of 250, 500, and 1,000 ng were processed similarly and assayed in duplicate. Fluoro-

metric readings were taken on an Aminco-Bowman spectrophotofluorometer at an excitation of 355 nm and an emission of 455 nm.

Statistics. Data were analyzed using Student's *t* and the Wilcoxon rank sum test. Linear regression coefficients were calculated by method of least squares (22).

RESULTS

Perfusion studies. Group A and group B animals received, respectively, cationic or native BSA, 25 mg i.v., followed by an isolated left kidney perfusion of identical anti-BSA antibody. This experimental technique only results in immune complex formation if the pathogenic mechanism of the model studied is in situ complex formation, since it is unlikely that circulating immune complexes are formed with this technique.

Capillary wall staining for sheep IgG (Fig. 1A) and BSA (Fig. 1B) at an intensity of 3–4 was present in the left kidneys of all group A animals within 10 min of the antibody perfusion. Upon reestablishment of the circulation, prominent capillary wall granular C3 was detected (Fig. 1B inset). In contrast, glomeruli in group B animals were consistently negative for sheep IgG, BSA, and C3 (Fig. 1C). This confirms the hypothesis that the capillary wall deposits observed are in fact produced by an in situ mechanism. All right control kidneys had prominent BSA in the capillary walls at an intensity identical to that seen in left kidneys, since BSA was delivered via an intravenous injection. However, no IgG or C3 was present in the right kidney at any time.

In vitro inhibition study. An in vitro inhibition study was utilized to demonstrate protamine's ability to block the binding of cationic BSA to the GBM. Incubation of GBM with protamine sulfate above 0.25 mg/ml diminished the subsequent uptake of ^{125}I-cationic BSA by the GBM compared to uptake in controls preincubated with NRS or PBS (Fig. 2).

Immunofluorescence and electron microscopy. In addition to the described in vitro demonstration of

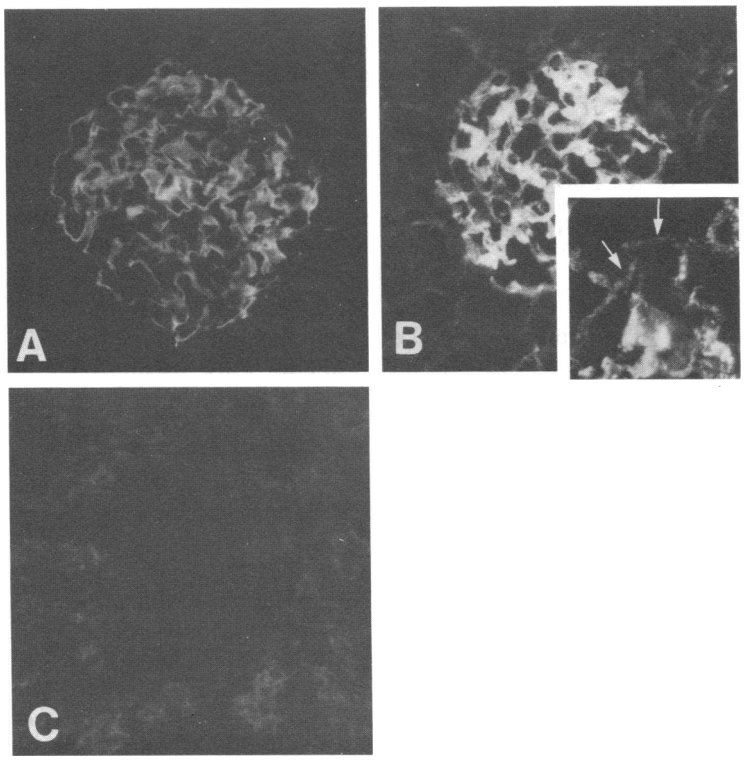

FIGURE 1 Representative immunofluorescence micrographs of glomeruli from left kidneys of group A (A, B) and group B (C) animals. (A) Sheep IgG is present in a diffuse capillary wall pattern after systemic injection of cationic BSA 25 mg i.v. followed by left renal artery perfusion with sheep anti-BSA antibody (×200). (B) Glomerulus from same animal as in A showing diffuse staining for BSA (×200). Inset: Higher magnification showing distinctly granular rabbit C3 along the capillary wall after restoration of the renal circulation (×504). (C) No deposits of IgG, BSA, or C3 were present in glomeruli from left and right kidneys of group B animals injected with native (anionic) BSA (×200).

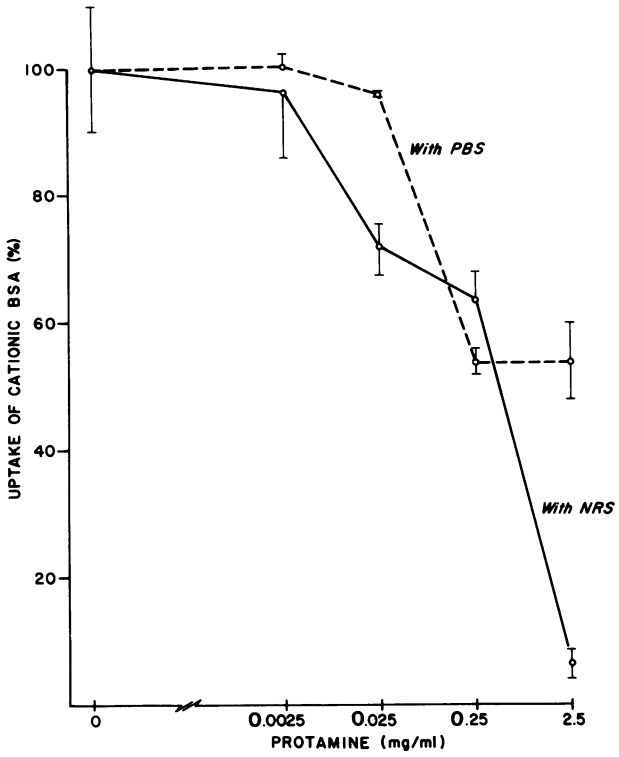

FIGURE 2 Preincubation of GBM with protamine ≥ 0.25 mg/ml inhibited the subsequent uptake of ^{125}I-cationic BSA by GBM ($P < 0.05$). This effect was more marked ($P < 0.001$) at high protamine concentrations when normal rabbit serum (NRS) rather than PBS was used to dilute the protamine to its final concentration.

protamine-induced inhibition of cationic BSA uptake by the GBM, we demonstrated the ability of protamine to bind to anionic sites in vivo in the rabbit glomerulus. 15 min after a 50-mg i.v. injection of protamine sulfate, spherical regularly arranged electron densities, ~20 nm in diam and 60 nm apart (Fig. 3) were observed along the lamina rara interna and lamina rara externa. They were also noted in the mesangium and occasionally along the surface of epithelial cells. These findings in the rabbit are strikingly similar to the appearance of anionic sites in the rat (10, 11, 23) and mark the documentation of these sites in this species. It should be noted that protamine sulfate's ability to bind to glomerular anionic sites is transient; protamine binding could not be demonstrated ultrastructurally in the rabbit glomerulus 24 h after the previous protamine dose.

All rabbits in group I developed typical membranous nephropathy, characterized by diffuse granular capillary wall deposits of IgG and C3 at week 2, increasing to maximal intensity in most cases by week 3. In comparison, the rabbits of group II treated with protamine sulfate had significantly fewer capillary wall deposits of IgG and C3 at the end of 2 and 3 wk of injection (Figs. 4 and 5). Cationic BSA could often be detected by immunofluorescence at the end of week 2, but decreased in intensity by the end of week 3, presumably because its binding sites had been covered by anti-BSA antibody. Rabbit albumin was never observed in the glomerular capillary wall, but was present as reabsorption droplets in proteinuric animals.

Frequent large subepithelial deposits associated with foot process effacement were typically seen in group I animals by the end of 2 and 3 wk of injection. In comparison, foot process effacement was infrequent in group II animals and subepithelial deposits, if present at all, were very small (Fig. 6). Although protamine generally impeded the development of capillary wall deposits in group II animals, three distinct patterns of response were observed. The majority of group II animals (9 of 18) had sparse or no capillary wall deposits by immunofluorescence and electron microscopy at week 2, with little or no progression at week 3. In 3 of 18 rabbits, similar sparse deposits were present at week 2, but progression to heavy capillary wall deposition occurred by week 3. An unexpected pattern developed in the remaining 6 animals. In these, mild to severe subepithelial deposits present at the end of week 2 showed either marked or total resolution 1 wk later despite continued injections of cationic BSA. Fig. 7 documents the most dramatic example of this protamine-induced resolution seen in our laboratory. It should be noted that this pattern of improvement was never observed in control animals injected with cationic BSA alone (group I).

In summary, all deposits in group I and group II animals were qualitatively similar, being typically epimembranous. In both groups, these lesions were characterized by capillary wall BSA, IgG, and C3, and ultrastructurally appeared as subepithelial dense deposits with foot process effacement. No mesangial or subendothelial deposits were observed in either group. The sole distinction between groups I and II was a quantitative one ($P < 0.001$ by Student's t test and by the Wilcoxon rank sum test).

The morphologic changes in biopsies from group III and IV animals (heparin and diphenhydramine control groups) were identical to those observed in group I. Immunofluorescence scoring of the capillary wall deposits seen in these groups is summarized in Fig. 8. Renal biopsies from group V rabbits after 6 wk of injection of protamine alone showed rare foot process effacement, mild vacuolization of tubular cells and occasional small platelet and fibrin aggregates in capillary lumens. These findings demonstrate a lack of important structural abnormalities in kidneys after the prolonged administration of protamine in the rabbit.

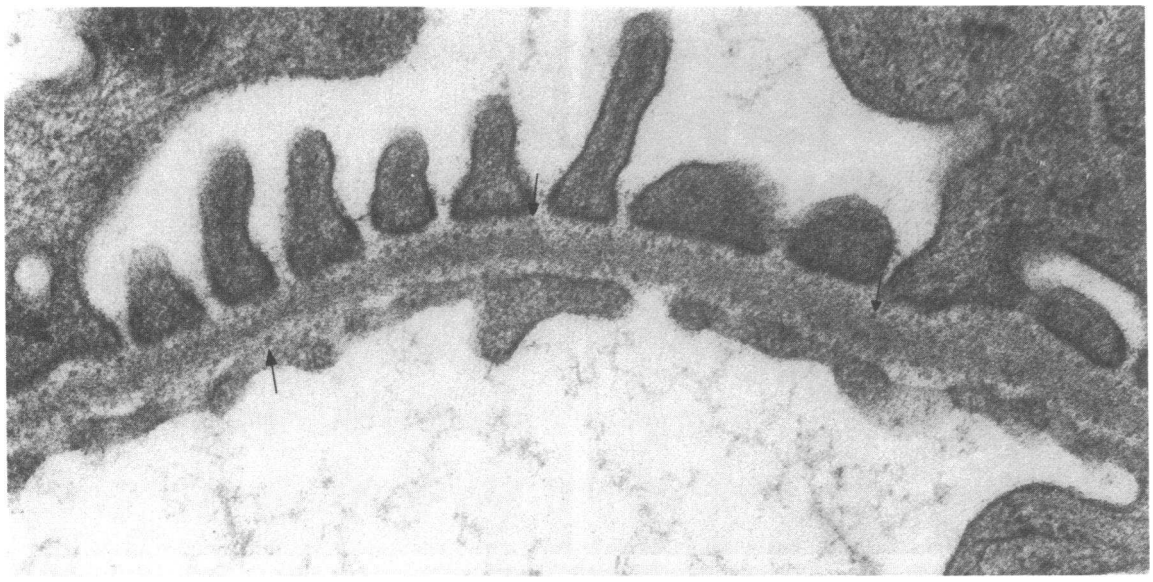

FIGURE 3 Electron micrograph documenting protamine's ability to bind to anionic sites in the rabbit glomerulus. Spherical electron densities (arrows) regularly arranged along the lamina rare interna and externa of the GBM seen 15 min after an intravenous injection of protamine sulfate, 50 mg. Anionic sites were not visualized after intravenous injections of normal saline (×68,575).

Laboratory measurements. In addition to protamine's amelioration of the renal lesion, there was also a marked reduction in albuminuria in group II animals. All rabbits had <15 mg urine albumin excretion in a 24-h period prior to injection as measured by radial immunodiffusion. By the end of 3 wk of injections, urine albumin excretion in group II was only one-fifth that seen in group I (124±55 mg vs. 632±150 mg) (Fig. 9). Serum creatinine rose in both groups to 1.1±0.1 mg/dl during the course of injections. The difference between the level of serum creatinine prior to immunization and that measured at the end of 3 wk of injection did not reach statistical significance in any group and is best explained by increased muscle mass in normally growing rabbits.

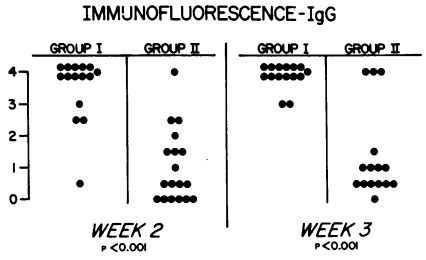

FIGURE 4 Semiquantitative grading of capillary wall IgG in individual animals after 2 and 3 wk of injections of cationic BSA alone (group I) or cationic BSA and protamine (group II). Capillary wall deposits were significantly fewer in animals receiving protamine at the end of both time periods ($P < 0.001$).

Total serum IgG and specific anti-BSA antibody were measured to ensure that protamine did not impede the development of epimembranous lesions via immunosuppression. During the course of injections, total serum IgG rose to a maximum of 1,089±189 and 1,117±116 mg/dl in groups I and II. Specific anti-BSA antibody peaked at 73.2±33.5 and 45.2±14.5 μg BSA/ml undiluted serum in groups I and II after 2 wk of injection. Although there was no significant difference ($P > 0.05$) in total serum IgG and specific anti-BSA antibody between the two groups at any point in time, there was a tendency for the protamine-treated group to have slightly lower total serum IgG and anti-BSA antibody levels than control animals. To examine whether this statistically nonsignificant trend in the protamine group had an effect on the development of immune deposits in the kidney, we compared the specific anti-BSA antibody levels of all the protamine-treated animals at the end of 2 and 3 wk of injection with the intensity of the capillary wall IgG seen on biopsy (Fig. 10). There was no correlation between the intensity of the deposits seen on biopsy and the magnitude of the immune response as measured by the antigen binding capacity test. In fact, the protamine-treated animal with the highest specific anti-BSA antibody level (which was more than twice as high as the control group mean), had only trace deposits of IgG

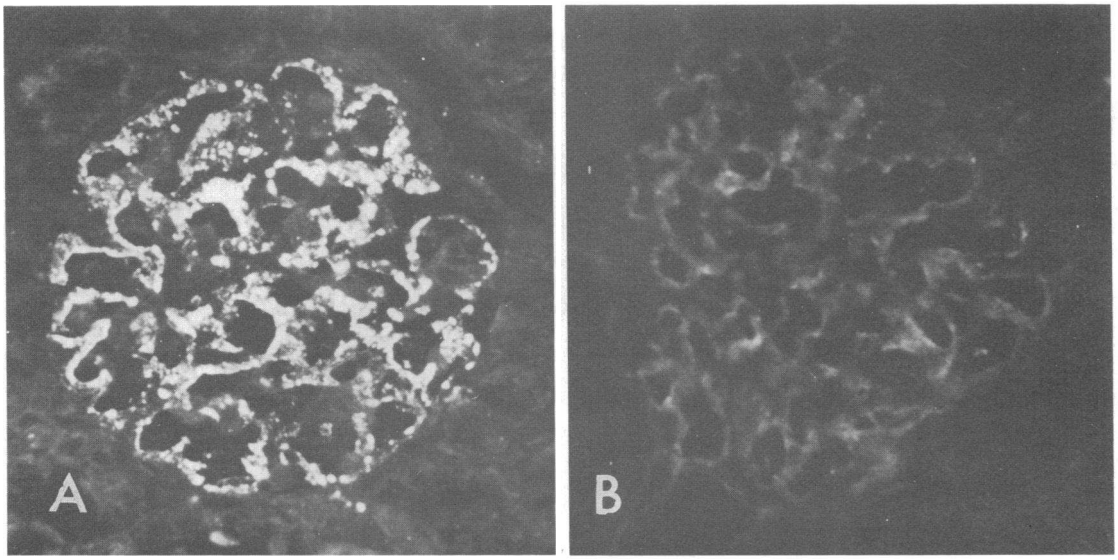

FIGURE 5 Immunofluorescence microscopy of renal biopsies at the end of 3 wk of injections. (A) Diffuse granular capillary wall deposits of IgG are present in a glomerulus from a rabbit receiving cationic BSA alone (group I, ×200). (B) Sparse capillary wall deposits are seen in a glomerulus from a rabbit receiving protamine and cationic BSA (group II, ×200).

in the capillary walls. In addition, two of four protamine-treated animals with heavy capillary wall IgG had the lowest levels of anti-BSA antibody measured in any of the groups. At the end of 3 wk of injection, serum C3 levels in group I were 99% and in group II 104% of those measured before immunization ($P > 0.05$). No significant differences ($P > 0.05$) were seen in the serologic or urinary measurements presented above among groups I, III, and IV rabbits. No increases in albuminuria or serum creatinine occurred during the 6 wk of injections in group V rabbits.

Circulating immune complex size. No qualitative

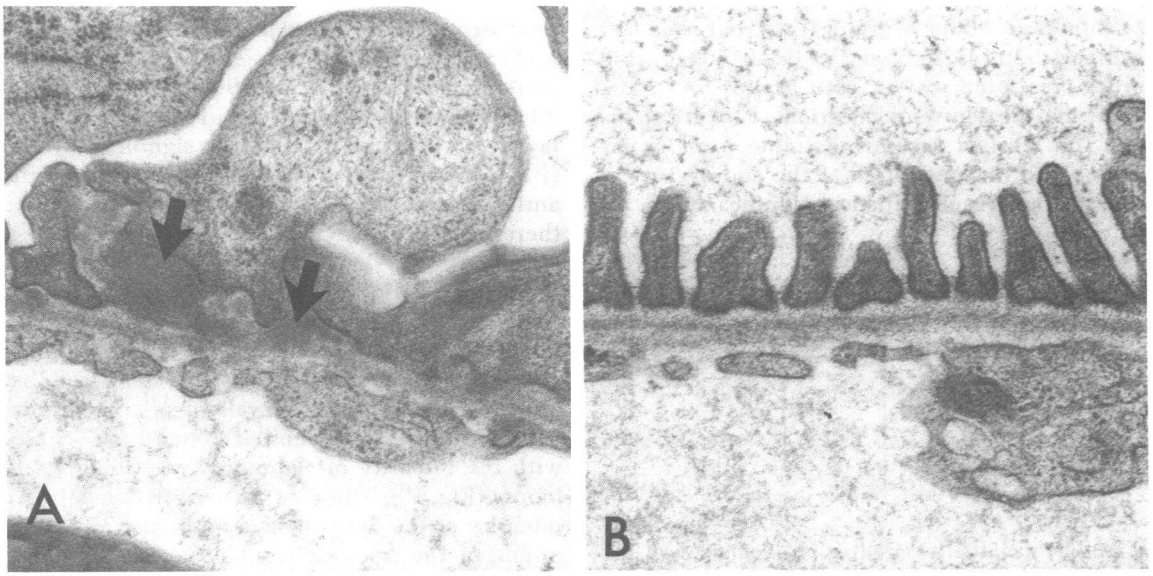

FIGURE 6 Ultrastructural appearance of renal biopsies after 3 wk of injection. (A) In group I animals, frequent dense subepithelial deposits (arrows) and foot process effacement are present (×24,800). (B) In group II, in contrast, absence of subepithelial deposits and normal foot process architecture is typical of rabbits demonstrating a beneficial response to protamine (×31,300).

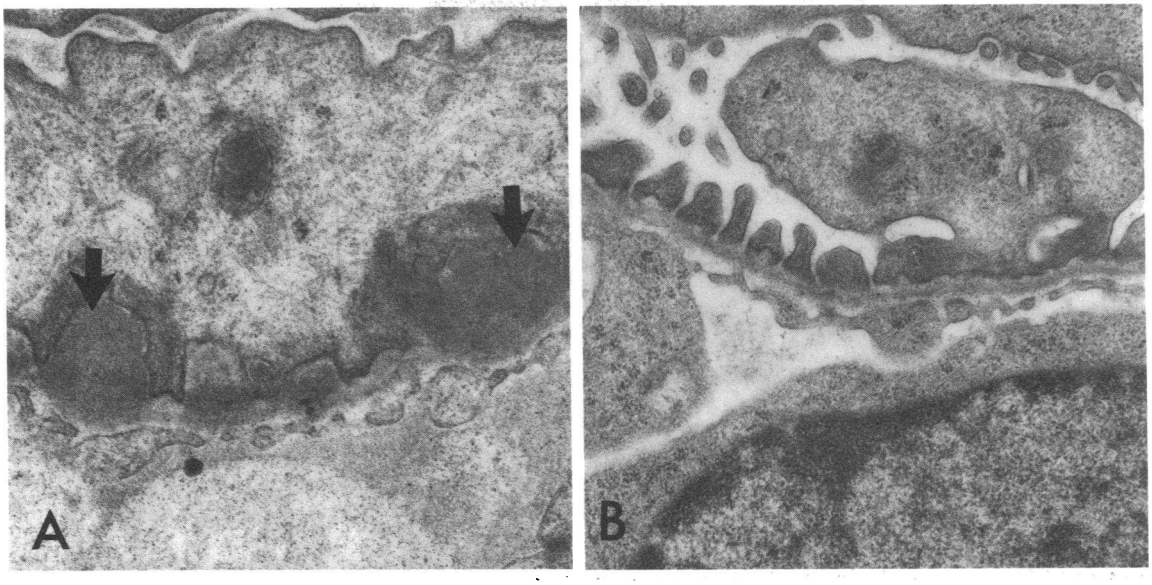

FIGURE 7 Renal biopsies from a rabbit in group II who failed to show a beneficial response to protamine at the end of 2 wk of injection. (A) Subepithelial dense deposits (arrows) and complete foot process effacement are present at the end of week 2 (×15,000). (B) Glomerular capillary wall from the same rabbit as in A, 1 wk later. Despite continued injections, the deposits have disappeared and the normal architecture of the foot processes has been restored (×18,850).

differences were seen in immune complex size between animals in groups I and II. At the end of 2 wk of injections, no complexes heavier than 500,000 D were found as previously reported in this model (9).

Whole kidney histamine content. Measurements were done in kidneys from three rabbits each in groups I and II. Homogenized whole kidney histamine content was comparable in groups I and II (577.3±41.1 and 653.7±23 ng/g, $P > 0.05$).

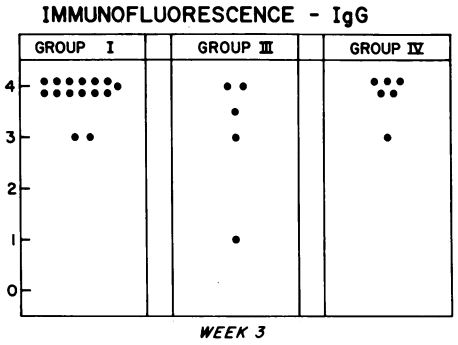

FIGURE 8 Semiquantitative grading of capillary wall IgG deposits in individual animals after 3 wk of injections of cationic BSA alone (group I), cationic BSA plus heparin (group III) and cationic BSA plus diphenhydramine (group IV). There were no significant differences in the quantity or quality of capillary wall deposits among these three groups ($P > 0.05$).

Effect of protamine on serum levels of cationic BSA. Serum levels of cationic BSA were measured in rabbits with saline or protamine pretreatment at var-

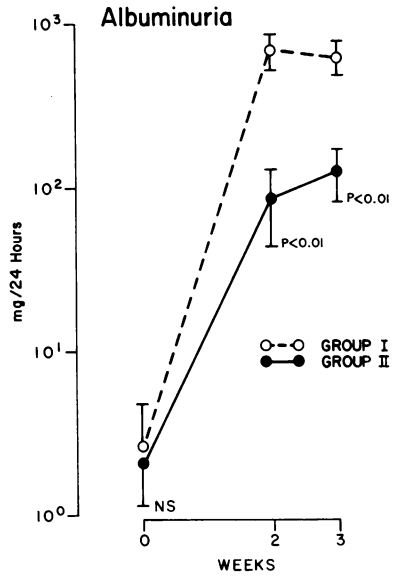

FIGURE 9 Significantly less urine albumin excretion occurred in the group receiving protamine and cationic BSA (group II) compared to the group receiving cationic BSA alone (group I) at the end of 2 and 3 wk of injection ($P < 0.01$).

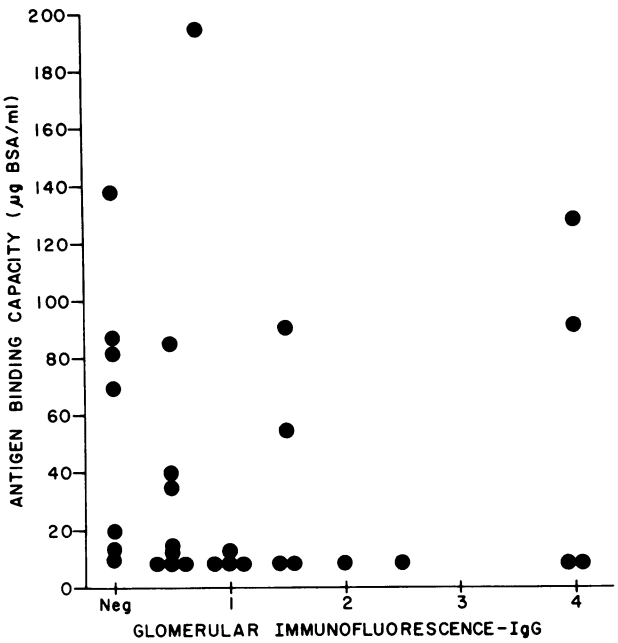

FIGURE 10 The height of the specific anti-BSA antibody response is plotted against the intensity of IgG in the glomerular capillary wall after 2 and 3 wk of injections of cationic BSA and protamine. There is no correlation ($r = 0$) between the level of the antibody response and the severity of glomerular deposits.

ious time intervals after an intravenous injection of ^{125}I-cationic BSA. Serum levels of ^{125}I-cationic BSA tended to be slightly higher in the protamine-treated group, but this trend did not reach statistical significance ($P > 0.05$). At no time were cationic BSA serum levels lower in the protamine- than in the saline-pretreated group.

DISCUSSION

Based on information gathered from studies of acute and chronic serum sickness (7, 8), most forms of immune complex glomerulonephritis (with the exception of antiglomerular basement membrane disease), have been presumed to be the result of the nonspecific deposition of circulating immune complexes in the glomerular filter. Historically, the factors considered important for renal immune complex localization have included immune complex size (24), antibody avidity (25, 26), serum immune complex levels (27), activity of the phagocytic reticuloendothelial system (28, 29), vasoactive amine activity (30), and hemodynamic factors (27, 30, 31). The nature of the inciting antigen, and the structural, chemical and physical properties of the glomerulus were not initially thought to play a major role in the development of glomerulonephritis.

In the past 10 years, evidence has been rapidly accumulating for a second pathogenic mechanism in glomerulonephritis. Van Damme et al. (2) and, independently, Couser et al. (3) demonstrated that the subepithelial deposits of Heymann's nephritis could be reproduced in the rat in the absence of circulating immune complexes. They suggested that these immune deposits were the result of in situ complex formation. At about the same time, elegant experiments demonstrated the presence of anionic sites in the rat glomerulus (10, 11, 23). Subsequently, work from our laboratory showed that the intravenous injection of a charge modified cationic species of BSA produced membranous nephropathy in the rabbit (9). We proposed that the subepithelial deposits observed were the result of in situ complex formation. In this case, anionic sites in the glomerular capillary wall acted as negatively charged receptors for the injected cationic antigen.

In the present study, using an ex vivo isolated perfused kidney technique, we showed that cationic BSA, IgG, and C3 can deposit in the kidney sequentially and form immune complexes in situ. In addition, we demonstrated the importance of a cationic antigen for this to occur, since similar deposits could not be produced using the native anionic (pI = 4.9) antigen. We are aware of criticisms of the perfused kidney technique raised by some investigators (32). Our alteration of the standard technique introduced by Hoyer et al. (33) involved administration of the antigen by a peripheral intravenous injection. This modification exposed the kidney to the antigen in a physiologic carrier (blood) rather than in perfusate. In addition, it shortened renal ischemia and anesthesia time. Biopsy of the contralateral control kidney, demonstrated that this modification was valid, since only intravenously injected BSA, and not perfused IgG or endogenous C3 could be identified in it by immunofluorescence microscopy. For these reasons, we feel this method has a physiologic advantage over the standard technique. We were not able to demonstrate in situ complex formation with native BSA as previously reported in the rat by Fleuren et al. (5). While species differences must be considered in this apparent contradiction, the longer ischemia time necessary for the perfusions of Fleuren et al. may also have played a role.

The discovery of electrical charge as a new pathogenic factor for the development of immune complex glomerulonephritis may have important clinical implications. Up to the present time, the treatment of glomerulonephritis has been approached by manipulation of the immune system with the use of immunosuppressant drugs and recently by the addition of plasmapheresis. In man and experimental animals, nonimmunologic therapies such as anticoagulants and

antihistaminics have not consistently met with success. In this study, we showed that an electrostatic attraction between the fixed anionic sites of the glomerular capillary wall and the injected circulating cationic antigen was primarily responsible for the development of membranous nephropathy in this model. We then tested whether protamine sulfate, a small, freely filterable, highly cationic, relatively nonimmunogenic molecule would compete with cationic antigen for binding at the anionic sites of the glomerular capillary wall and delay the development of membranous nephropathy. Protamine effectively prevented or delayed the onset of membranous nephropathy in 15 of 18 rabbits ($P < 0.001$) injected with cationic BSA. In addition, the heavy albuminuria seen in the animals receiving cationic BSA alone was not present in the animals receiving protamine and cationic BSA ($P < 0.01$). Protamine's beneficial effects were not mediated by immunosuppression. Total serum IgG and specific anti-BSA antibody were not significantly different ($P > 0.05$) in any of the groups studied. In addition, no significant difference ($P > 0.05$) was noted in serum C3 levels, circulating immune complex size, or serum levels of cationic BSA after pretreatment with protamine or saline. The beneficial effects of protamine were not related to its weak anticoagulant property or to its theoretical ability to deplete tissue histamine. The capillary wall lesion observed in rabbits receiving cationic BSA alone was unaltered by the concomitant administration of heparin or diphenhydramine. In addition, homogenized whole kidney histamine content was not significantly different in animals receiving cationic BSA alone or with protamine. Protamine sulfate was shown to bind transiently to the anionic sites of the rabbit glomerulus by electron microscopy in vivo and to inhibit GBM uptake of subsequently administered radiolabeled cationic BSA in vitro ($P < 0.05$). This evidence supports the hypothesis that the beneficial effects of protamine on the morphologic and functional changes in this model of membranous nephropathy are due to its ability to interfere with the first step of in situ complex formation: the binding of the exogenous cationic antigen to anionic sites in the glomerular capillary wall.

During the course of our experiments with protamine sulfate, we unexpectedly discovered that in addition to protamine's ability to impede the development of subepithelial deposits, it was also capable of enhancing the clearance of subepithelial deposits from the GBM in those animals where lesions did develop early in the injection schedule (i.e., after 2 wk of injection of cationic BSA and protamine). 6 of 18 protamine-treated animals failed to show a beneficial response at the end of 2 wk of injection, and had numerous subepithelial deposits and foot process effacement. Despite continued cationic BSA injections, 1 wk later, resolution of foot process effacement and clearance of subepithelial deposits were demonstrated. It should be noted that this pattern of resolution was never observed in animals receiving cationic BSA alone in this study, or in other studies during our extensive use of this model. We feel that inhibition of cationic BSA binding by protamine is an unlikely sole explanation for the dramatic disappearance of these deposits, although any proposed mechanism at this time would be speculative. In this regard, it is possible that protamine chemically interacts with subepithelial immune deposits to disrupt the antigen-antibody bond and facilitate rapid clearance from the GBM. Alternatively, it is possible that the GBM-protamine electrostatic attraction is stronger than that between the GBM and the immune deposit, and under some conditions, protamine may be able to displace or dissolve the immune complex and propel it into the urinary space. It has been suggested by Sharon et al. (34) that rats with protamine-heparin complexes may exhibit increased glomerular visceral epithelial cell phagocytosis. Protamine may play a role in this proposed phagocytic enhancement, thereby rapidly clearing the subepithelial space of foreign materials. Another possibility is that protamine in some way affects the formation or metabolism of anionic sites along the GBM, with the net result of reducing the number of available sites. This could occur by either decreased formation of sites or increased breakdown of sites. Further work will be necessary to clarify the mechanism of action of protamine with regard to the disappearance of subepithelial deposits.

The effect of small polycations on the subsequent binding of charged macromolecules has been studied by other groups. Kelley and Cavallo (35) showed that the perfusion of protamine sulfate directly into the renal artery of Sprague-Dawley rats increased the subsequent uptake of perfused anionic ferritin into the lamina rara externa. More recently, Barnes et al. showed that the injection of polyethyleneimine (a low molecular weight polycation) also increased the subsequent uptake of anionic ferritin in the rat glomerulus (36). Bertolatus and Hunsicker (37) demonstrated diminished glomerular uptake of lysozyme and cationized ferritin after an intravenous infusion of hexadimethrine. In addition, this group has also shown that negatively charged polyacrylamide gels pretreated with hexadimethrine bound less subsequently administered cationic ferritin than did untreated gels (37). Ours is the first demonstration of the usefulness of a competing polycation in the prevention of glomerulonephritis in an active animal model.

Polycation infusions have been reported to cause foot process effacement (38), proteinuria (39), and at

high concentrations, glomerular thrombosis and renal failure (40). These reports would seem to conflict with ours in that we did not demonstrate important permanent alterations in glomerular structure by electron microscopy or in glomerular function by measurements of urine albumin and serum creatinine even after 6 wk of twice-daily protamine injections. Although we are unable to ensure that minor transient changes did not occur, our findings indicate that protamine administration in this model did not result in physiologically notable alterations, probably because of the small amount of protamine likely to have actually reached the kidney after intravenous injections. It is entirely possible that higher systemic doses of protamine than those used in this study would have caused adverse effects similar to those previously described.

To our knowledge, this is the first time that competition between cationic molecules has played a role in the therapy of experimental immune complex disease by specific nonimmunologic interference with the in situ pathogenic mechanism. It is our hope that this report will stimulate new approaches to the therapy of immune glomerulonephritis in man.

Note added in proof. Support for the lack of protamine's nephrotoxicity, at the doses we used, has been provided by Vahaskari et al. (1982. *Kidney Int.* **22:** 127–135), who showed that abnormal proteinuria did not occur in protamine-injected rats until the dose exceeded 25 μg/g body wt. Our rabbits received less than the critical dose.

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